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Immunomodulatory and antiviral effects of Poly I:C-LC, CL 24673 and Ampligen were studied. All three agents stimulated the reticuloendothelial system function of mice, although the timing of stimulation varied from one drug to other. Poly I:C-LC caused transient inhibition followed by stimulation; CL 246738 caused stimulation between days 2 and 4 post treatment and Ampligen caused transient stimulation. Poly I:C-LC had the strongest effect on phagocytic cells; CL 246738 had a transient effect observed only on day 2 post treatment while Ampligen had minimal effect. Poly I:C-LC had mild suppressive effect on PFC responses when given before antigen and some augmenting effect when given after antigen. Suppression and augmentation appeared to be due to alteration in the splenic nucleated cell population. CL 246738 was without significant effect in this assay. Both Poly I:C-LC and Ampligen caused a reduction in the proportion of T and B lymphocytes, although the time of this reduction differed for the two drugs. CL 246738 is without effect on this parameter. Both CL 246738 and Ampligen potentiated macrophage extrinsic antiviral activity. All three drugs enhanced NK-cell activity and serum interferon levels, although Poly I:C-LC had most profound effect on these functions.

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BIOLOGY OF IMMUNOMODULATORS

Annual Report

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July 1987

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SUMMARY

We have compared effects of Poly I:C-LC, CL 24673 and Ampligen on clearance and organ localization of radiolabelled sheep erytorocytes (SRBC); peritoneal, splenic and liver cell phagocytosis; antibody PFC responses; splenic and liver natural killer (NK) cytotoxicity; specific T cell cytotoxicity; enumeration of lymphoid cell subpopulations; macrophage antiviral activity and serum interferon levels. We have also examined the effect of these agents on resistance to herpes, Punta Toro and Banzi virus infections.

All three agents tested were capable of stimulating the blood clearance (reticuloendothelial) function of mice, although the timing of stimulation varied from one drug to other. Poly I:C-LC caused transient inhibition (day 2) followed by stimulation (day 7); CL 246738 caused stimulation between days 2 and 4 post treatment and Ampligen caused transient stimulation (day 2). Poly I:C-LC had the strongest effect on phagocytic cells and this effect persisted for at least 7 days in the peritonium. CL 246738 had a transient effect observed only on day 2 post treatment while Ampligen had minimal effects on phagocytic activity. Poly I:C-LC had mild suppressive effect on PFC responses when given before antigen and some augmenting effect when given after antigen. Suppression appeared to be due to a dilution of the PFC resulting from expansion of splenic nucleated cell population. Similarly, augmentation also appeared to be due to an enlargement of spleens in treated animals. CL 246738 was without significant effect in this assay. Both Poly I:C-LC and Ampligen may cause a reduction in the proportion of T and B lymphocytes, although the time of this reduction may differ for the two drugs. CL 246738 is without effect on this parameter. Both CL 246738 and Ampligen potentiated macrophage extrinsic antiviral activity: cells from CL 246738 treated mice were about 100 times more effective than control macrophages and cells from Ampligen treated mice were about 10 times more effective. All three drugs enhanced the NK cytotoxicity and serum interferon levels, although Poly I:C-LC had most profound effect on these parameters.

All three drugs can afford somne protection in the HSV-1 induced pneumonitis model when given prophylactically on D -2 or on the day of virus challenge. However, none of the drugs was effective in treating influenza pneumonitis. Poly I:C-LC and CL 246738 were also effective as prophylactic treatment in Punta Toro induced hepatitis. However, only Poly I:C-LC was effective in treating HSV-1 induced hepatitis. Poly I:C-LC given prophylactically was most effective in treating Banzi virus induced encephalitis (90-100% protection) whereas CL 246738 and Ampligen were relatively less effective (20-60% protection). None of the drugs were effective against HSV-1 induced encephalitis.

[illegible]; antiviral agents; macrophages; lymphocytes; Banzi virus; encephalitis; [illegible] herpes; hepatitus; (KT)

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985)

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I. PROBLEM UNDER INVESTIGATION

This study was designed to evaluate the multifacited effects of selected immunoenhancing drugs on specific and nonspecific components of the immune system which are of importance in resistance to and recovery from viral infections. We have examined the effect of treatment schedule on various in vitro and in vivo immune parameters. The immune parameters examined included:

A. In Vitro / Ex Vivo Evaluation of Nonspecific Elements Affecting the Course of Viral Disease:

1. Macrophage antiviral cytotoxicity
2. Natural killer (NK) cell cytotoxicity
3. Production of interferons (IF)
4. Clearance of radiolabeled erythrocytes from blood and their localization in various organs
5. Phagocytosis by peritoneal, splenic and liver macrophages

B. In vitro / Ex Vivo Evaluation of Specific Elements Affecting Resistance to and Recovery from Viral Diseases:

1. Antibody responses to T-dependent antigens
2. T cell cytotoxicity
3. Alterations in T and B lymphocyte populations and subpopulations (e.g., T helper or suppressor cells)

C. Evaluation of Host Resistance to and Recovery from Viral Infections:

II. BACKGROUND

Members of the military are exposed to a variety of viruses which often result in infections leading to serious illness or death. Although they can sometimes be protected by active immunization, this approach is not always practical due to difficulties in producing either attenuated or killed vaccines which are both safe and immunogenic. In addition, vaccines are of little value in the therapy of active viral infections. Therefore, alternative approaches have been explored. One approach has been the development of antiviral drugs. While these drugs have been effective, in some situations, their use has been hampered by their toxic side effects and limited range of activity.

Another approach to prevention and treatment of viral infections has been immunotherapy. Although immunotherapy with classical agents has had some success, it has also been plagued by toxicity problems. However, the recent development of chemically defined or synthetic immunostimulants with low toxicity and broad spectrum activity has made this approach more appealing. These immunostimulants have been used alone and in combination with vaccines in prophylaxis or with antiviral compounds in therapy.

While there are numerous reports of the efficacy of the newer generation immunostimulants, the experimental approaches utilizing these compounds have varied, thus, making an objective analysis of their comparative efficacy difficult. In addition, since the cellular components of the immune system that need to be stimulated will vary depending on the pathogenic features of the virus, it is essential that the mode of action of immunostimulating drugs be defined. Because the comparative efficacy and mode of action of many immunostimulants have not been fully explored their use has been mostly empirical. A more rational approach for the selection of appropriate drugs for use in prophylaxis or therapy requires 1) a comparison of the efficacy of various agents under the same experimental conditions and with the same panel of tests and 2) a better understanding of their modes of action.

Most immunostimulants possess a unique set of immunomodulating features and provide varying degrees of benefit to the infected host. The beneficial effects imparted by these immunostimulants will largely depend on the tissue site and degree of virus infection. For example, it may be desirable to have elevated levels of interferon in some tissue sites during a particular time of infection but not during others. This may be particularly relevant in some arenavirus infections (e.g., lymphocytic choriomeningitis virus; LCMV) in which interferon can have detrimental effects (1, 2). Likewise, activated NK cells and macrophages may result in immunopathologic damage which can contribute to the disease process (3). Because of these complexities, the choice of immunomodulating agents, their dose, time

and frequency of administration require careful consideration of the immunopathologic features of infection. This is only possible if one is able to identify the spectrum of changes induced by a particular drug.

By virtue of their position at sites of initial infection and wide distribution in major organs of the body, macrophages and NK cells and their soluble mediators (e.g., PG, IL, MAF and IF) are thought to be of prime importance in resistance to a number of intracellular pathogens. Thus, for many viral infections macrophage function has been shown to be an important factor in determining the course of the disease (4-7). For example, in herpesvirus infections both resistance to virus replication within macrophages (intrinsic resistance) and macrophage antiviral effects on other virus infected cells (extrinsic antiviral activity) may be significant determinants in host resistance. (8)

In addition to macrophages, another cell type which plays a significant role in primary resistance to virus infection is the NK cell (9-11). Unlike the cytotoxic T lymphocyte, this cell destroys virus infected cells without prior sensitization and thus quickly limits virus dissemination (11). A positive correlation between genetically determined resistance to virus lethality and the level of NK cell augmentation has been observed in both murine cytomegalovirus and herpes simplex virus infections (12,13).

A variety of soluble mediators may be released following the administration of various immunostimulants. Some of these mediators may have a negative effect on the immune system while others may have a positive effect. For example, prostaglandins may have a detrimental effect due to their negative feedback control on cellular functions (14-16). In contrast, interferon has a beneficial role in inhibition of virus replication as well as in the augmentation of cellular components of the immune system. While each type of interferon (i.e. alpha, beta and gamma) possess the ability to induce the antiviral state in cells, gamma interferon may be more important since it also regulates various immune functions (17-19).

There are a number of reports on the use of macrophage activators in the treatment of infectious diseases. Most notably, these compounds have been used prophylactically to enhance nonspecific resistance by direct activation of macrophages and NK cells or via the the induction of soluble mediators. For example, inoculation of mice with Escherichia coli endotoxin, Staphylcoccus aureus, BCG, or the lipoidal amine (CP-20,961) enhances resistance to influenza virus through the induction of interferon and/or the activation of macrophages and NK cells (20-23). Similar effects against herpesviruses, Newcastle disease, encephalomyocarditis, vesicular stomatitis, and Junin viruses were observed after treatment with various immunostimulants (24-30). Likewise, inoculation of mice with P. acnes induced protection against various hemoprotozoans (31-34).

In addition to their effects on macrophages and NK cells immunostimulating agents also affect elements of the specific immune response. Since both antibody and cell mediated immune responses are involved in resistance to and recovery from viral infections, immunostimulating drugs have been used in combination with whole, and subunit viral vaccines in an attempt to enhance their immunogenicity (35, 36). Use of immunostimulants may be particularly valuable in those situation in which cloned vaccines are available, since these antigens are poor immunogens.

Unfortunately, selection of appropriate immunostimulants to use with vaccines has been somewhat empirical. This is due to the variety of cellular targets on which immunostimulants can act, and the paucity of information concerning the their effects on these targets. For example, some immunostimulants, or the soluble mediators released in response to them, may selectively potentiate B cells, or suppressor or helper T cells which may influence the quantity of antibody produced following vaccination (37-39). In contrast, other immunostimulants may preferentially augment cytotoxic T cells which can have profound effects on recovery from viral disease but have little impact on resistance to viral infection.

In summary, immunoenhancing drugs can exert their effect by interacting with one or more of the cellular components of the immune system. These components are affected either directly, or indirectly through the action of soluble mediators. The ultimate outcome of such drug interactions will depend upon which of the various components is influenced. Therefore, the judicious use of immunoenhancing drugs, together with vaccines in prophylaxis or in the therapy of viral infections of military importance, requires a thorough understanding of their relative effects on the numerous components of the immune system.

While the prophylactic use of immunopotentiating substances has been widely studied, their therapeutic value has not been well documented. In addition, the comparative efficacy and mode of action of various immunostimulants against a variety of infectious agents (especially those of military significance) has not been adequately examined.

Our studies will provide the comparative data on a spectrum of immunological parameters for various immunoenhancing drugs. These data will provide a more scientific basis for the use of various immunoenhancing agents, either alone or in combination with vaccines or antivirals, in the effective treatment of viral diseases of importance to the military.

III. EXPERIMENTAL APPROACH

In this project each immunoenhancing drug was studied in two phases. During the first phase we examined the effects of selected drugs on a variety of components in the immune system. In the second phase we applied the knowledge gained from the initial phase to design experimental protocols to evaluate the clinical potential of these drugs. The studies were performed in animal models of human viral disease.

Phase I consisted of experiments designed to characterize the effects which selected immunostimulants exerted on the nonspecific or specific components of the immune system. Drugs were administered to C3H/HeN mice, intraperitoneally (i.p.), intravenously (iv.) or orally and appropriate cells or fluids obtained at selected intervals. The cells were examined in vitro for a variety of effector functions and their characteristic surface markers. The fluids were examined for the presence of soluble mediators. The effects oftime of treatment was also assessed.

Phase II studies were designed to assess the effects of immunostimulants on resistance to and recovery from viral infection. Based on the immunological profiles from phase I and the pathogenesis of the viral agents under study, appropriate drugs were selected for either prophylaxis or therapy. Animals were examined for their ability to survive challenge with lethal doses of infectious agent. These experiments were performed using murine models of influenza virus herpesvirus, Punta Toro and Banzi virus infections. Lung, liver and brain infections were studied. The following animal models were employed.

Influenza Virus Pneumonitis: The virus used in these studies is a mouse adapted H3N2 strain of influenza A virus (Aichi). When 2-10 LD_{50} of this strain is administered intranasally into six to seven week old C3H/HeN mice, death, due to interstitial pneumonia, occurs in five to seven days. Virus is found only in the lungs and mice eventually die of pneumonia.

HSV-1 Pneumonitis: The virus used in these studies is a human isolate (VR3 strain) of type 1 herpes simplex virus (HSV-1) obtained from Dr. Andre Nahmias (Emory University, Atlanta, GA). Intranasal inoculation of three to five week old C3H/HeN mice with 2-10 LD_{50} of virus results in a fulminant pneumonitis and adrenalitis. Death occurs five to eight days following infection. A unique aspect of this model is that encephalitis does not occur.

HSV-1 Encephalitis: The virus used to induce encephalitis is a human isolate (MB strain) of type 1 herpes simplex virus obtained from Dr. Richard Whitley (Univ. Ala, Birmingham, AL). Footpad inoculation of four week old C3H/HeN mice results in virus replication in the sciatic

nerve, spinal cord and brain. Mice die of encephalitis six to eight days after inoculation. Immunoperoxidase staining for viral antigen has been used to confirm this mode of virus dissemination.

HSV-1 Hepatitis: The MB virus strain was used to induce liver disease. When four to five week old C3H/HeN mice are inoculated intravenously with 2-10 LD_{50} of virus, the primary organ of initial infection is the liver. Viremia and dissemination to a number of other organs follows liver infection and death results five to seven days post infection.

Banzi Virus Encephalitis: The seed virus used in these studies was obtained from Dr. C.J. Peters (USAMRIID, Fort Detrick, MD). Working stocks of virus are prepared from suckling mouse brains. When inoculated subcutaneously, this virus replicates in peripheral lymphoid tissue and is carried to the spleen. Viremia results 2-4 days post infection and the virus enters the brain. Encephalitis is observed 6-8 days post infection. Death ensues 8-10 days following the administration of as little as 10 p.f.u.

Punta Toro Hepatitis: The seed virus (Adames strain) used in our studies was initially prepared by Dr. D. Pifat (USAMRIID, Fort Detrick, MD). Working stocks of this virus are prepared by passage of cloned virus in Vero cells. Cloned virus was obtained from Dr. W. Sidwell (Univ. Utah) following enrichment and further characterization of the virus prepared by Pifat. Subcutaneous inoculation of 10^4 p.f.u. of our seed virus into 4 week old C3H/HeN mice results in hepatocellular necrosis and death 4-7 days post infection. A unique aspect of this virus infection is the tissue tropism which appears to be restricted to the liver and spleen even in the presence of high levels of circulating virus in the blood.

IV. RESULTS

During the first year of this contract, we have focused our studies primarily on the comparative effects of Ampligen, CL 246738 and Poly I:C-LC on a number of immunological parameters, although, we have also begun investigations on other drugs. The parameters examined included: in vivo clearance and organ localization of radiolabelled sheep erytorocytes (SRBC); peritoneal, splenic and liver cell phagocytosis; antibody plaque-forming cell PFC) responses; splenic and liver natural killer (NK) cytotoxicity; specific T cell cytotoxicity; enumeration of lymphoid cell subpopulations; macrophage antiviral activity and serum interferon levels. We have also examined the effect of these agents on resistance to herpes, Punta Toro and Banzi virus infections.

In Vivo Clearance and Organ Localization of Erythrocytes

Tables 1-4 contain data on the effect of Poly I:C-LC, Ampligen and CL 246738 on clearance rate of SRBC from circulation and their localization in liver, spleen and lung. The clearance rates are presented as T/2 and K-values. An increase in K-value reflects an increase in the rate of clearance and consequently a decrease in the half-life (T/2) of SRBC in circulation. Also listed in the tables are alpha values which represent clearance rates normalized for mouse body, spleen and liver weights. Thus, increased alpha values also represent increased clearance rates. Organ localization is presented as number of SRBC per mg wet tissue.

Two days after iv. administration of Poly I:C-LC there was a reduction in the clearance rate of SRBC which was not due to alterations in the body or organ weights (Table 1). This reduction was apparently due to reduced localization in liver which is the major organ for clearance of particulate material from circulation. The decreased clearance rate represents a real increase in the SRBC half-life, since essentially all of the labelled cells were shown to be free (i.e., not internalized) in the circulation. Also, the increased localization of SRBC in the spleen and the lung was not due to increased plasma volume and represents SRBC that have been phagocytosed. Furthermore, the alterations listed above were not due to the action of lysine-carboxymethylcellulose component of Poly I:C-LC (see Table 1, quarterly report #2).

The reduction in clearance rate observed on day 2 following Poly I:C-LC injection was not seen 4 days after treatment, and at this time, there was an increased localization in liver. By 7 days after treatment there was an increased clearance rate which could be accounted for by increased liver weight and increased hepatic localization. By 14 days after treatement no differences were observed between control and treated animals. Thus, Poly I:C-LC

causes a transient impairment (day 2) of the reticuloendothelial system followed by a slight enhancment (day 7). These effects were apparently due to the effect of the drug on liver function.

In contrast to Poly I:C-LC, both CL 246738, given orally, and Ampligen, given iv., increased blood clearance rate 2 days after treatment which appeared to be due to increased liver localization (Tables 2 & 3). The effect of CL 246738 was still evident on day 4 after treatment (Table 2) while no effect of Ampligen was seen at this time (Table 3). By 7 days after treatment neither CL 246738 nor Ampligen had any effect. The effect of CL 246738 given iv., even approaching toxic doses, was negligible (Table 4). Thus, Ampligen and CL 246738 have similar effects (stimulation) on clearance rate whereas Poly I:C-LC has an opposite effect (inhibition). In all cases these effects are transient.

In summary, all three agents tested are capable of stimulating the blood clearance function (reticuloendothelial function) of mice, although the timing of stimulation varies from one drug to other. Poly I:C-LC causes transient inhibition (day 2) followed by stimulation (day 7), CL 246738 causes stimulation between days 2 and 4 posttreatment and Ampligen causes transient stimulation (day 2).

Peritoneal, Splenic and Liver Cell Phagocytosis

Peritoneal, splenic and liver cell phagocytic activity was assessed as described in our third Quarterly Report. Data from representative experiments are shown in Figures 1-4. Poly I:C-LC given two days prior to test increased the phagocytic acitvity of peritoneal cells as evidenced by the increased flourescnece intensity (i.e., greater number of internalized flourescent bacteria) in the drug treated group (Figure 1). Furthermore, drug treatment also increased the proportion of phagocytic cells in the peritoneal population (15% vs 37% for highly phagocytic cells; channels 128-255). These effects were still evident on day 4 after treatment (Figure 2), began to wane on day 7 (Figure 3) and were near normal by day 14 (Figure 4). Results on the effects of Poly I:C-LC on splenic cells were somewhat variable: in one experiment there was evidence for an increase in the number of phagocytic cells (8% vs 16% for highly phagocytic cells) without increased flourescence intensity whereas in another experiment there was no alteration. In a preliminary experiment, liver phagocytic cells from Poly I:C-LC treated animals exhibited an inhibition of activity and a decrease in the number of phagocytic cells (31% vs 11% for highly phagocytic cells), which is consistent with the results obtained in the clearance experiments described above.

Treatment with CL 246738 also increased the activity and number of phagocytic peritoneal cells (26% vs 39% for highly phagocytic cells) 2 days after treatment. However, by 4 days after treatment no significant differences were observed between controls and treated

groups. Similarly, CL 246738 had little effect on splenic or liver phagocytic cells when administered 2 or 4 days prior to assay.

Treatment with Ampligen did not increase the number of phagocytic cells in the peritoneal population 2 days after treatment, although in one experiment there was some indication of increased phagocytic activity. No effect was observed 4 days after treatment. Similarly, Ampligen had no apparent effect on the number or activity of splenic or liver phagocytic cells at any time tested.

In summary, Poly I:C-LC had the strongest effect on phagocytic cells and this effect persisted for at least 7 days in the peritonium. CL 246738 had a transient effect observed only on day 2 post treatment while Ampligen had minimal effects on phagocytic activity.

Antibody Plaque-Forming Cell Responses

The effect of Poly I:C-LC on antibody PFC was somewhat variable. When given before antigen, some suppression of plaque forming cells per million splenocytes was observed (Table 5). However, this reduction was apparent for only IgG PFC when the total number of PFC was calculated. This discrepancy apparently due to expansion of the splenic mononuclear cell population after drug treatment. When administered after antigen, Poly I:C-LC either did not alter the plaque forming cell response or, sometimes, it caused a slight enhancement, as exemplified by the IgM PFC per spleen (Table 5).

Generally, CL 246738 did not have any effect on the plaque forming cell response whether given before or after antigen (Table 6). The significance of reduction in the IgG PFC per 10^6 will have to be reevaluated. However, Ampligen showed some similarities with Poly I:C-LC in that it suppressed IgG PFC per 10^6 when given before antigen and enhanced the IgM PFC per spleen when given after antigen (Table 7).

In summary Poly I:C-LC had mild suppressive effect on PFC responses when given before antigen and some augmenting effect when given after antigen. Suppression appeared to be due to a dilution of the PFC resulting from expansion of splenic nucleated cell population. Similarly augmentation also appeared to be due to an enlargement of spleens in treated animals. CL 246738 was without significant effect in this assay.

Splenic and Liver Natural Killer Cells

The effects of Poly I:C-LC, CL 246738 and Ampligen on splenic and liver NK cytotoxicity was assessed as described in our second and third Quarterly Reports and the data is presneted in Tables (8-10). Poly I:C-LC, given 2 days prior to assay, was able to augment splenic

NK activity and this augmentation persisted for at least 4 days (Table 8). However, on day 7 or 14, no significant augmentation in spleen NK cell activity was observed. Although we have not as yet examined the effects of Poly I:C-LC, given 2 and 4 days prior to assay, on liver NK cell activity, the data in Table 8 demonstrate that Poly I:C-LC may be able to augment liver NK activity for at least 14 days after treatment. These observations will be confirmed shortly.

CL 246738 was similar to Poly I:C-LC in being able to augment splenic NK activity day 2 and day 4 after treatment (Tables 9). However, this drug was not able to augment liver NK activity using the same treatment schedules. We are currently repeating these observations and investigating whether CL 246738 will augment splenic and liver NK cell activity after 7 or 14 days.

Ampligen like Poly I:C-LC and CL 246738 was able to augment splenic NK cell activity 2 days after treatment, however, unlike Poly I:C-LC and CL 246738 no significant augmentation was observed 4 days after treatment (Table 10). Also, like CL 246738, Ampligen had little effect on liver NK activity (Table 10).

In summary, all three drugs, Poly I:C-LC, CL 246738 and Ampligen were able to augmennt splenic NK activity, but the effect was transient, lasting less than 7 days for Poly I:C-LC and 4 days for CL 246738 and Ampligen. Only Poly I:C-LC augmented liver NK cytotoxicity.

Specific T Cell Cytotoxicity

We have recently begun studies on the effects of drugs on specific T cell cytotoxicity. The design of these experiments is as follows. Animals are treated with drug, sacrificed various times thereafter and their spleen cells are incubated *in vitro* with mitocycin-C treated allogeneic cells for 4-5 days. Specific T cell cytotoxicity of sensitized splenocytes is then measured using ^{51}Cr-labeled target cells with the same H-2 haplotype as the stimulator cells.

Our initial studies indicate that treatment with Poly I:C-LC, 2 days prior to *in vitro* sensitization, resulted in a significant decrease in specific T cell cytotoxicity whereas treatment with CL 246738 had no significant effect (Table 11). We are currently repeating these studies and expanding these interesting observations.

Enumeration of Lymphoid Cell Subpopulations

Splenic lymphocyte subpopulations were enumerated by treating cells from control and drug treated animals with fluorescenated antibodies and determining the number of fluorescent cells on the flow cytometer. The antibodies used included: anti-IgG, for B cells; monoclonal anti-Thy-1, for T cells; monoclonal anti-Ly-1 and anti-Ly-2 for the

Ly1+2+, Ly1+2- and Ly 1-2+ T cell subpopulations. The data obtained to date are presented in Table 12. As can be seen in this table, treatment with Poly I:C-LC on day -2, -4 or -14 had no effect on the splenic lymphocyte subpopulations. Treatment with Poly I:C-LC on day -7 may decrease the number of B cells, T cells and T cell subpopulations but this observation remains to be confirmed. CL 246738 treatment did not appear to have an effect on the lymphocyte subpopulations. In contrast, treatment with Ampligen may decrease the proportion of B cells, T cells and T cell subpopulations but a definite conclusion must await our repeating these experiments.

In summary, Poly I:C-LC and Ampligen may both cause a reduction in the proportion of T and B lymphocytes, although the time of this reduction may differ for the two drugs. CL 246738 is without effect on this parameter.

Macrophage Antiviral Activity

Macrophage extrinsic antiviral activity was assessed by culturing peritoneal macrophages from control or drug treated animals with HSV-1 infected Vero cells for 3 days and measuring the reduction in virus titers. As can be seen from Table 13, macrophages from control animals were able to reduce the virus titers by 1.76 logs while macrophages from CL 246738 and Ampligen treated mice reduced titers by 3.61 and 2.88, respectively. Thus, both CL 246738 and Ampligen potentiated macropohage extrinsic antiviral activity: cells from CL 246738 treated mice were about 100 times more effective than control macrophages and cells from Ampligen treated mice were about 10 times more effective.

Interferon Levels

Serum interferon levels following treatment with Poly I:C-LC, CL 246738 or Ampligen were examined on days, 1, 2, 3, 4 and 8 following drug treatment using a VSV plaque reduction assay. As can be seen in Figure 5, the response to all three drugs peaks 1 day after treatment and is significantly reduced by day 3. Interferon concentrations reach background levels by day 8. Moreover, Poly I:C-LC given iv. was a more effective interferon inducer than Ampligen given by the same route or CL 246738 given orally.

Resistance to Herpes, Punta Toro and Banzi Virus Infections

The ability of Poly I:C-LC, CL 246738 and Ampligen to enhance antiviral resistance was examined in murine models of pneumonitis, hepatitis and encephalitis. The results of these experiments are presented in Figures 6-14.

Pneumonitis Models

Two viruses which induce murine pneumonitis were employed, influenza (Aichi strain) and HSV-1. In these models 10 LD_{50} of virus was administered intranasally and mortality monitored for 21-30 days. As can be seen in Figure 6, 100 ug of Poly I:C-LC, administered on the day of or 2 days prior to challenge with influenza virus, was unable to protect the mice. In contrast, 70-80% protection was obtained in the HSV-1 pneumonitis model when Poly I:C-LC was given on the same schedule. However, Poly I:C-LC was ineffective if given therapeutically 1 day after virus challenge.

Like Poly I:C-LC , CL 246738 and Ampligen were unable to protect mice challenged with influenza virus (Figures 7 and 8). However, CL 246738 treatment gave some (30%) protection when given on the day of virus challenge and moderate (50%) protection when given prophylactically on D -2 (Figure 7). Ampligen was equally effective as CL 246738 in protecting when given prophylactically on D -2 and perhaps slightly more effective when given on the day of virus challenge (Figure 8).

In summary, all three drugs can afford somne protection in the HSV-1 induced pneumonitis model when given prophylactically on D -2 or on the day of virus challenge. However, none of the durgs was effective in treating influenza pneumonitis.

Hepatitis Models

Two viruses which induce murine hepatitis were employed in our studies, HSV-1 (given iv.) and Punta Toro (given ip.). In these models 10 LD_{50} of virus was administered by the route indicated above and mortality monitored for 21-30 days. Poly I:C-LC was effective (70% protection) in treating HSV-1 induced hepatitis when given prophylactically on D -2 but it was apparently ineffective when given on the day of virus challenge (Figure 9). Similarly, Poly I:C-LC was able to protect (100 %) mice challenged with Punta Toro virus when given prophylactically.

Unlike Poly I:C-LC, neither CL 246738 nor Ampligen were able to protect against HSV-1 induced hepatitis even when given prophylactically (Figures 10 & 11). However, CL 246738 was completely protective in the Punta Toro virus model. Ampligen has not yet been tested in this model.

In summary, both Poly I:C-LC and CL 246738 can be used to treat Punta Toro induced murine hepatitis using a prophylactic protocol. However, only Poly I:C-LC is effective in treating HSV-1 induced hepatitis.

Encephalitis Models

Two models of encephalitis were employed, one which uses HSV-1 (given via the foot pad) and the other which uses Banzi virus (given ip.). Poly I:C-LC was able to give good protection (90%-100%) only in the Banzi virus model and only when given prophylactically or on the day of virus challenge (Figure 12). Only slight protection (20%) was observed when the drug was given therapeutically 1 day after challenge. CL 246738 also protected in the Banzi virus model when given prophylactically but the protection was not as good (20 % when given on D -0 and 60 % when given on D -2) as was seen with Poly I:C-LC (Figure 13). No protection was observed when CL 246738 was given therapeutically. In the Banzivirus model, the efects of Ampligen were similar to CL 246738. (Figure 14)

In summary, Poly I:C-LC given prophylactically was the best in treating Banivirus induced encephalitis (90-100% protection) while CL 246738 and Ampligen were less effective (20-60% protection). None of the drugs was effective against HSV-1 induced encephalitis.

V. CONCLUSIONS

The ge ierated in the first year of this study has resulted in the following conclusions:

1. Poly I:C-LC alters the ability of mice to clear particulate material from circulation, uptake by liver and spleen in a variable manner: it is inhibitory on day 2 post treatment but stimulatory on day 7.

2. Poly I:C-LC stimulates the phagocytic activity of peritoneal macrophages

3. Poly I:C-LC appears inhibitory for antibody PCF response when given before antigen but is stimulatory when given after.

4. Poly I:C-LC enhances serum interferon levels

5. Poly I:C-LC augments NK cell cytotoxicity

6. Poly I:C-LC, given prophylactically, is protective in HSV, but not influenza, induced pneumonitis, HSV and Punta Toro virus hepatitis and Banzi virus encephalitis.

7. CL 246738 transiently stimulates <u>in vivo</u> clearance and <u>in vitro</u> phagocytic function of peritoneal cells.

8. CL 246738 enhances serum interferon levels.

9. CL 246738 augments NK cell cytotoxicity.

10. CL 246738 given prophylactically, is protective in HSV, but not influenza, induced pneumonitis, Punta Toro virus hepatitis and Banzi virus encephalitis.

11. Ampligen transiently stimulates <u>in vivo</u> clearance.

12. Ampligen may affect antibody PFC response in a manner similar to Poly I:C-LC.

13. Ampligen enhances serum interferon levels.

14. Ampligen augments NK cell cytotoxicity and its effect may be longer lasting than that of CL 246738.

15. Ampligen given prophylactically, is protective in HSV, but not influenza virus induced pneumonitis and Banzi virus encephalitis.

VI. RECOMMENDATIONS

In the forthcoming year we will complete profile as planned and continue building a profile on newer agents. We have already begun studies on a pyrimidinone (ABPP) and Isoprinosine. We have also been investigating therapeutic efficacy of immunomodulators on Friend virus inuduced leukemia, a retroviral disease in mice. This aspect was added to the protocol in consultation with the scientific project officer.

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Table 1. Clearance and tissue localization of SRBC following Poly I:C-LC treatment.

TREATMENT		RBC/mg Tissue (x1000)			Phagocytic Index		
		Liver	Spleen	Lung	T/2 (min)	alpha Value	K Value
CONTROL	Mean	78.81	129.47	17.53	3.13	7.40	.1051
	Std. Dev.	11.39	39.73	10.40	.94	1.42	.0351
Poly IC-LC Day -2	Mean	27.99	223.19	74.70	13.40	4.80	.0280
	Std. Dev.	12.48	108.12	33.50	6.44	1.0[illegible]	.0150
	P-Value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Poly IC-LC Day -4	Mean	64.06	140.61	46.81	3.73	6.70	.0936
	Std. Dev.	23.53	52.02	23.38	1.85	1.64	.0319
	P-Value	<0.01	N.S.	<0.001	N.S.	N.S.	N.S.
Poly IC-LC Day -7	Mean	87.57	62.66	15.07	2.76	6.19	.1216
	Std. Dev.	14.11	15.66	11.44	.98	.85	.0389
	P-Value	<0.05	<0.001	<0.005	N.S.	<0.01	N.S.
Poly IC-LC Day -14	Mean	74.61	119.29	31.31	3.44	7.26	.1005
	Std. Dev.	12.82	39.55	13.17	1.37	.70	.0380
	P-Value	N.S.	N.S.	<0.001	N.S.	N.S.	N.S.

100 ug Poly I:C-LC was injected iv. on day -2, -4, -7 or -14 and tested for clearance on day 0.

Table 2. Clearance and tissue localization of SRBC following oral CL 246738 treatment.

TREATMENT		RBC/mg Tissue (x1000)			Phagocytic Index		
		Liver	Spleen	Lung	T/2 (min)	alpha Value	K Value
CONTROL	Mean	73.08	187.46	34.83	4.83	6.54	.0706
	Std. Dev.	10.81	60.45	19.56	1.64	.71	.0286
CL 246738 Day -2	Mean	95.08	121.11	10.60	2.43	7.45	.1283
	Std. Dev.	15.16	36.00	6.58	.47	.91	.0256
	p-Value	<0.001	<0.001	<0.001	<0.001	<0.005	<0.001
CL 246738 Day -4	Mean	88.23	103.35	15.64	3.05	7.14	.1152
	Std. Dev.	19.48	37.54	11.94	1.12	.77	.0462
	P-Value=	<0.02	<0.001	<0.005	<0.005	<0.05	<0.005
CL 246738 Day -7	Mean	96.39	200.88	46.75	5.04	7.08	.0642
	Std.Dev.	18.66	62.69	12.92	1.24	.7098	.0226
	p-Value	<0.005	N.S.	N.S.	N.S.	N.S.	N.S.
CL 246738 Day -14	Mean	90.85	201.51	37.30	4.59	7.21	.0672
	Std.Dev.	12.08	46.86	7.65	.75	.2760	.0124
	p-Value	<0.005	N.S.	N.S.	N.S.	N.S.	N.S.

4 mg CL246738 was administered orally on day -2, -4 , -7 or -14 and tested for clearance on day 0.

Table 3. Clearance and tissue localization of SRBC following Ampligen treatment.

TREATMENT		RBC/mg Tissue (x1000)			Phagocytic Index		
		Liver	Spleen	Lung	T/2 (min)	alpha Value	K Value
CONTROL	Mean	72.38	172.22	25.02	3.82	6.60	.0842
	Std. Dev.	12.50	41.66	11.35	1.10	.70	.0228
Ampligen Day -2	Mean	95.03	115.00	15.08	2.25	7.38	.1370
	Std. Dev.	11.15	50.67	10.47	.46	.48	.0321
	p-Value	<0.001	<0.001	<0.01	<0.001	<0.001	<0.001
CONTROL	Mean	70.94	157.07	28.53	3.88	6.69	.0847
	Std. Dev.	13.31	44.00	19.39	1.24	.89	.0266
Ampligen Day -4	Mean	84.17	130.14	21.50	3.31	6.52	.1443
	Std. Dev.	15.54	52.13	13.33	1.19	.74	.1658
	P-Value	<0.02	N.S.	N.S.	N.S.	N.S.	N.S.
CONTROL	Mean	75.65	157.81	21.86	3.46	6.69	.0893
	Std. Dev.	12.86	41.50	10.30	.75	.85	.0193
Ampligen Day -7	Mean	84.59	151.59	26.47	3.68	6.95	.0841
	Std. Dev.	7.53	53.83	8.55	.68	.80	.0149
	P-Value	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

100 ug Ampligen was injected iv. on day -2, -4 or -7 and tested for clearance on day 0.

Table 4. Clearance and tissue localization of SRBC following iv. CL 246738 treatment.

TREATMENT		RBC/mg Tissue (x1000)			Phagocytic Index		
		Liver	Spleen	Lung	T/2 (min)	alpha Value	K Value
CONTROL	Mean	74.82	131.77	23.68	3.90	6.52	.0910
	Std. Dev.	12.12	65.71	17.67	1.65	.84	.0378
CL 246738 Day -2	Mean	84.19	129.87	14.55	3.16	6.91	.1032
	Std. Dev.	10.23	56.17	16.19	1.00	.59	.0286
	P-Value	<0.05	N.S.	N.S.	N.S.	N.S.	N.S.
CONTROL	Mean	71.22	126.01	25.58	4.02	6.39	.0882
	Std. Dev.	11.25	62.73	19.44	1.73	.89	.0369
CL 246738 Day -4	Mean	70.86	157.14	26.74	3.79	6.64	.0755
	Std. Dev.	7.56	27.04	17.69	.81	.85	.0152
	P-Value	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

500 ug CL 246738 was injected iv. on day -2 or -4 and tested for clearance on day 0.

Table 5. Effect of Poly I:C-LC on antibody plaque forming cell (PFC) response.

Treatment		Cells/ Spleen x1000	IgM Plaques		IgG Plaques	
			PFC/10^6	PFC/Spl	PFC/10^6	PFC/Spl
Control	Geo. Mean	79	656	47,835	861	62,732
	Log Mean	1.86	2.82	4.68	2.94	4.80
	Std. Dev.	0.18	0.24	0.30	0.43	0.42
Poly I:C-LC Day -1	Geo. Mean	131	412	42,493	141	19,184
	Log Mean	2.12	2.62	4.63	2.15	4.28
	Std. Dev.	0.26	0.35	0.43	0.37	0.46
	p-Value	<0.001	<0.01	N.S.	<0.001	<0.001
Poly I:C-LC Day +1	Geometric	102	744	75,660	954	97,237
	Log Mean	2.01	2.87	4.88	2.98	4.99
	Std. Dev.	0.16	0.25	0.29	0.40	0.45
	p-Value	<0.01	N.S.	<0.05	N.S.	N.S.

Mice were injected iv. with 100 ug Poly I:C-LC either 1 day before or 1 day after i.p. immunization with $1x10^8$ SRBC and tested 5 days later. Results from 4 experiments were pooled for analysis.

Table 6. Effect of CL 246738 on antibody plaque forming cell (PFC) response.

Treatment		Cells/ Spleen x1000	IgM Plaques		IgG Plaques	
			PFC/10^6	PFC/Spl	PFC/10^6	PFC/Spl
Control	Geo. Mean	86	894	76,779	1,183	101,625
	Log Mean	1.93	2.95	4.89	3.07	5.01
	Std. Dev.	0.17	0.11	0.20	0.25	0.37
CL 246738 Day -1	Geo. Mean	88	975	85,566	592	51,971
	Log Mean	1.94	2.99	4.93	2.77	4.72
	Std. Dev.	0.12	0.18	0.17	0.28	0.32
	p-Value	N.S.	N.S.	N.S.	<0.05	N.S.
CL 246738 Day +1	Geometric	95	1,027	97,513	1,101	104,506
	Log Mean	1.98	3.01	4.99	3.04	5.02
	Std. Dev.	0.09	0.11	0.17	0.37	0.41
	p-Value	<0.001	N.S.	N.S.	N.S.	N.S.

Mice were given 4 mg CL 246738 either 1 day before or 1 day after i.p. immunization with $1x10^8$ SRBC tested 5 days later. Results from 2 experiments were pooled for analysis.

Table 7. Effect of Ampligen on antibody plaque forming cell (PFC) response.

Treatment		Cells/ Spleen x1000	IgM Plaques PFC/10^6	IgM Plaques PFC/Spl	IgG Plaques PFC/10^6	IgG Plaques PFC/Spl
Control	Geo. Mean	84	664	56,062	1,973	166,484
	Log Mean	1.93	2.82	4.75	3.30	5.22
	Std. Dev.	0.12	0.17	0.16	0.15	0.12
Ampligen Day -1	Geo. Mean	164	616	100,935	1,216	157,984
	Log Mean	2.14	2.79	5.00	3.09	5.20
	Std. Dev.	0.06	0.24	0.25	0.18	0.38
	p-Value	<0.001	N.S.	<0.05	<0.02	N.S.
Ampligen Day +1	Geometric	212	566	120,352	2,575	547,473
	Log Mean	2.33	2.75	5.08	3.41	5.74
	Std. Dev.	0.10	0.16	0.14	0.17	0.09
	p-Value	<0.001	N.S.	<0.001	N.S.	<0.001

Mice were injected iv. with 100 ug Ampligen either 1 day before or 1 day after i.p. immunization with $1x10^8$ SRBC tested 5 days later. Results from 2 experiments were pooled for analysis.

Table 8. Effect of Poly I:C-LC on splen and liver NK cytotoxicity.

	PERCENT CYTOTOXICITY					
	Spleen at E:T Ratios			Liver at E:T Ratios		
Treatment	100:1	50:1	25:1	100:1	50:1	25:1
Control	25	17	10	Not Tested		
Poly I:C-LC Day -2*	57	38	25			
Poly I:C-LC Day -4*	40	29	19			
Control	18	10	5	12	10	9
Poly I:C-LC Day -7$	25	13	3	50	54	50
Poly I:C-LC Day -14$	18	12	5	40	31	27

100 ug Poly I:C-LC was injected iv. on day -2, -4, -7 or -14 and tested for NK cytotoxicity on day 0.

*p-values <0.02 relative to control.

$p- values <0.02 relative to control for liver cells only. Spleen cels from drug treated animals were not significantly different from controls.

Table 9. Effect of CL 246738 on spleen and liver NK cytotoxicity.

Treatment		PERCENT CYTOTOXICITY					
		Spleen at E:T Ratios			Liver at E:T Ratios		
		100:1	50:1	25:1	100:1	50:1	25:1
Day -2	Cntrol	7	5	5	8	7	5
	CL-246738*	57	41	27	12	11	8
Day -4	Cntrol	20	14	9	2	2	1
	CL-246738*	65	60	49	15	12	9

4 mg CL 246738 was given orally on day -2 or -4 and tested for NK cytotoxicity on day 0.

*p-values <0.02 relative to control.

Table 10. Effect of Ampligen on spleen and liver NK cytotoxicity.

	PERCENT CYTOTOXICITY					
	Spleen at E:T Ratios			Liver at E:T Ratios		
Treatment	100:1	50:1	25:1	100:1	50:1	25:1
Control	19	14	12	9	8	7
Ampligen Day -2*	43	30	18	15	15	11
Ampligen Day -4	33	22	13	13	16	13

100 ug Ampligen was injected iv. on day -2 or -4 and tested for NK cytotoxicity on day 0.

*p-values <0.02 relative to control for spleen. Liver cells from drug treated animals were not lignificantly different from controls.

Table 11. Effect of Poly I:C-LC and CL 246738 on the induction of specific T cell cytotoxicity in splenocytes.

Treatment	% Cytotoxicity at E:T Ratios			
	100:1	50:1	25:1	12.5:1
Control	63	48	30	17
Poly I:C-LC Day -2*	24	9	6	3
Control	35	24	16	9
CL 246738 Day -2	27	18	9	4

100 ug Poly I:C-LC was injected iv. or 4 mg CL 246738 was administered orally on day -2 or -4 and tested on day 0 for <u>in vitro</u> induction of T cell cytotoxicity against allogeneic target.

*p-values <0.01 relative to control.

Table 12. Effects of Poly I:C-LC, CL 246738 and Ampligen on splenic lymphocyte subpopulations.

	% of Population				
Treatment	B Cells	T Cells	Ly1+2+ Cells	Ly 1+2- Cells	Ly 1-2+ Cells
Control	51+/-12	31+/-10	7+/-5	16+/-9	4+/-2
Poly I:C-LC Day -2 (#)	62+/-2	33+/-9	7+/-3	15+/-7	0+/-1
Poly I:C-LC Day -4 (#)	42+/-13	36+/-11	6+/-1	18+/-10	4+/-4
Poly I:C-LC Day -7 (*)	31	17	2	6	4
Poly I:C-LC Day -14 (#)	55+/-10	32+/-1	6+/-6	16+/-2	3+/-4
CL 246738 Day -2 (#)	57+/-13	27+/-7	6+/-8	13+/-3	9+/-4
CL 246738 Day -4 (*)	62	44	15	11	0
CL 246738 Day -7 (*)	40	17	0	11	6
Ampligen Day -2 (*)	35	18	3	7	1
Ampligen Day -4 (*)	36	20	2	8	3

100 ug Poly I:C-LC was injected iv. or 4 mg CL 246738 was administered orally on day -2 or -4 and tested on day 0.

(#) Differences are not significant

(*) Single experiment to be repeated.

Table 13. Effects of CL 246738 and Ampligen on macrophage antiviral activity.

Treatment	Virus Titer/ml	Log Reduction
None	1.9×10^7	-
Control	3.3×10^5	1.76
CL 246738	4.7×10^3	3.61
Ampligen	2.5×10^4	2.88
Poly I:C-LC	Not Tested	-

4mg CL 246738 was administered orally or 100 ug Ampligen was injected iv. on day -2 days and tested on day 0.

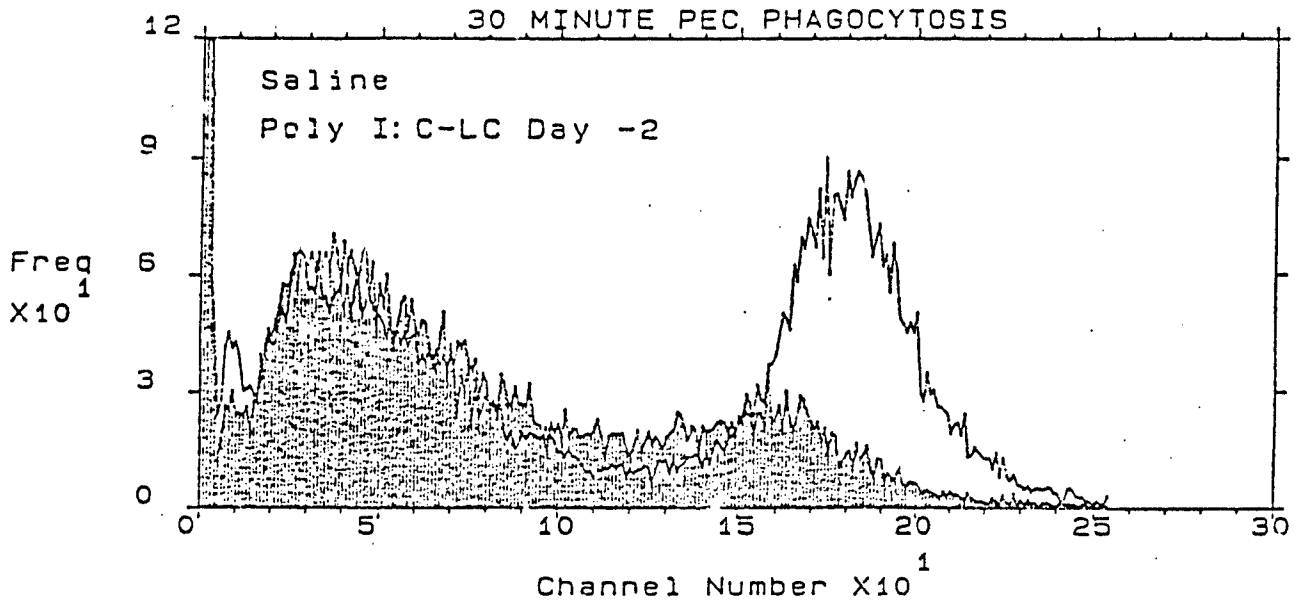

Figure 1. Flow Cytometric analysis of phagocytosis by peritoneal cells from mice treated with 100ug Poly I:C-LC on day -2.

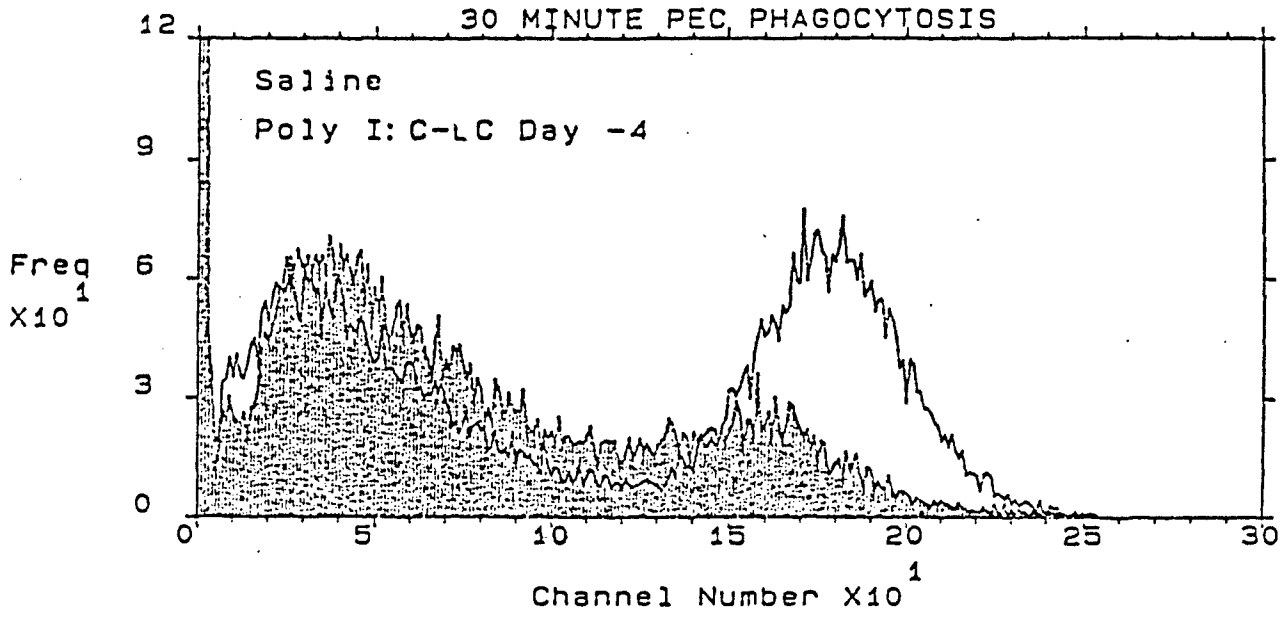

Figure 2. Flow Cytometric analysis of phagocytosis by peritoneal cells from mice treated with 100ug Poly I:C-LC on day -4.

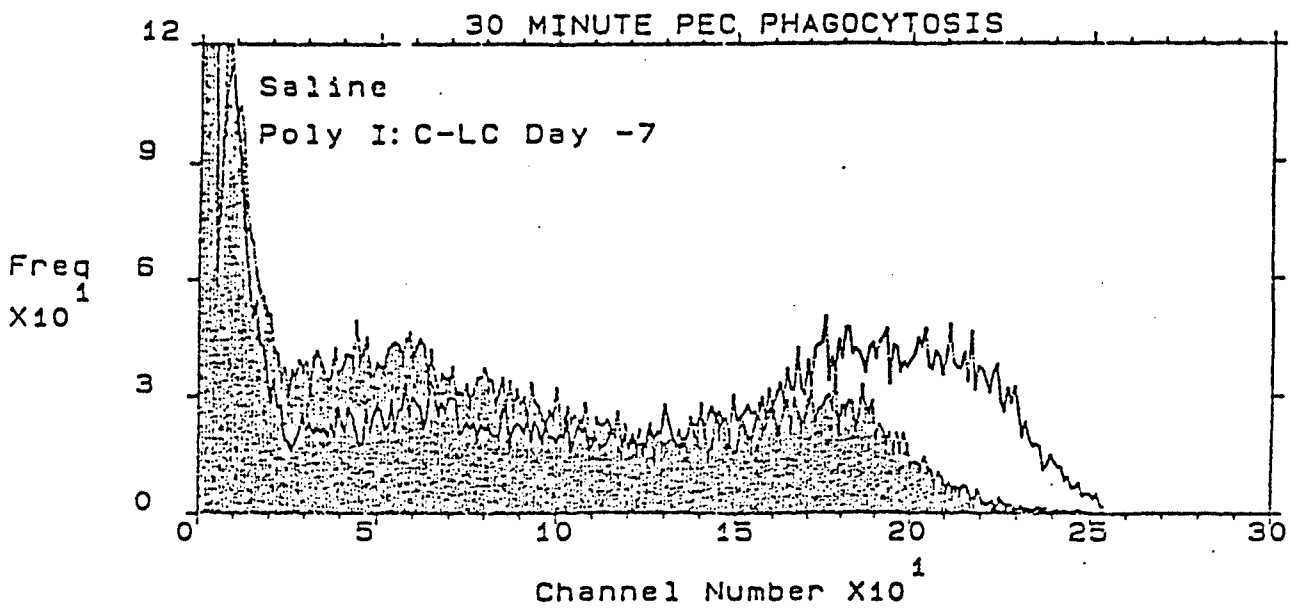

Figure 3. Flow Cytometric analysis of phagocytosis by peritoneal cells from mice treated with 100ug Poly I:C-LC on day -7.

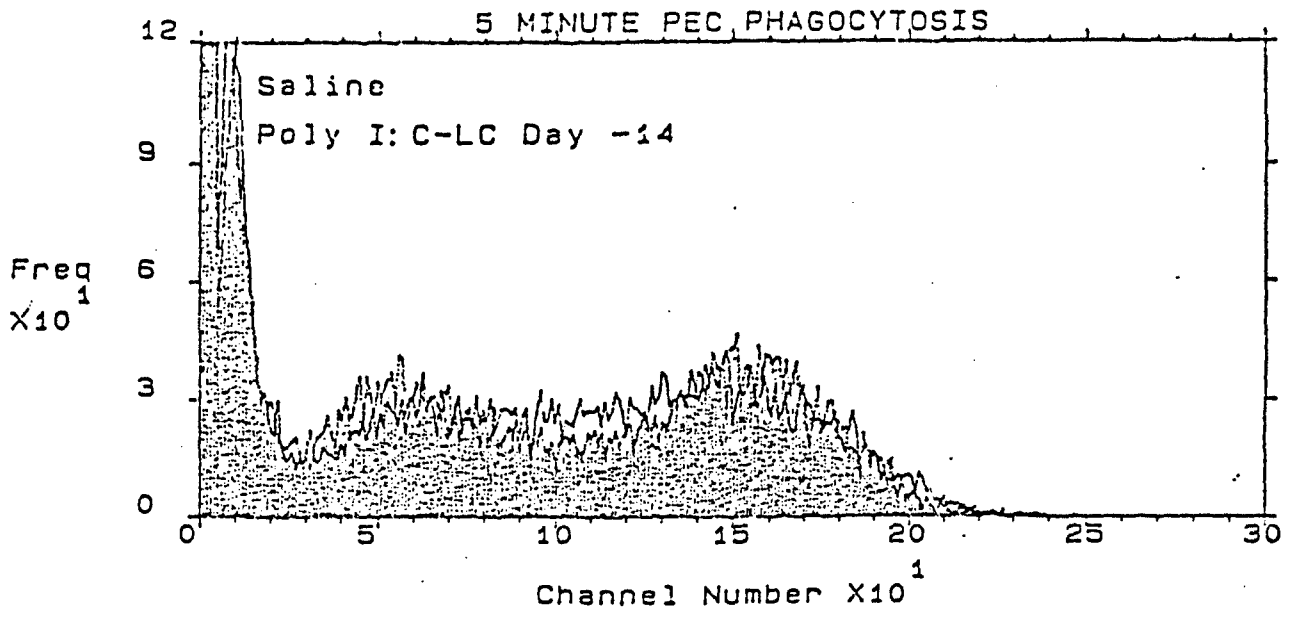

Figure 4. Flow Cytometric analysis of phagocytosis by peritoneal cells from mice treated with 100ug Poly I:C-LC on day -14.

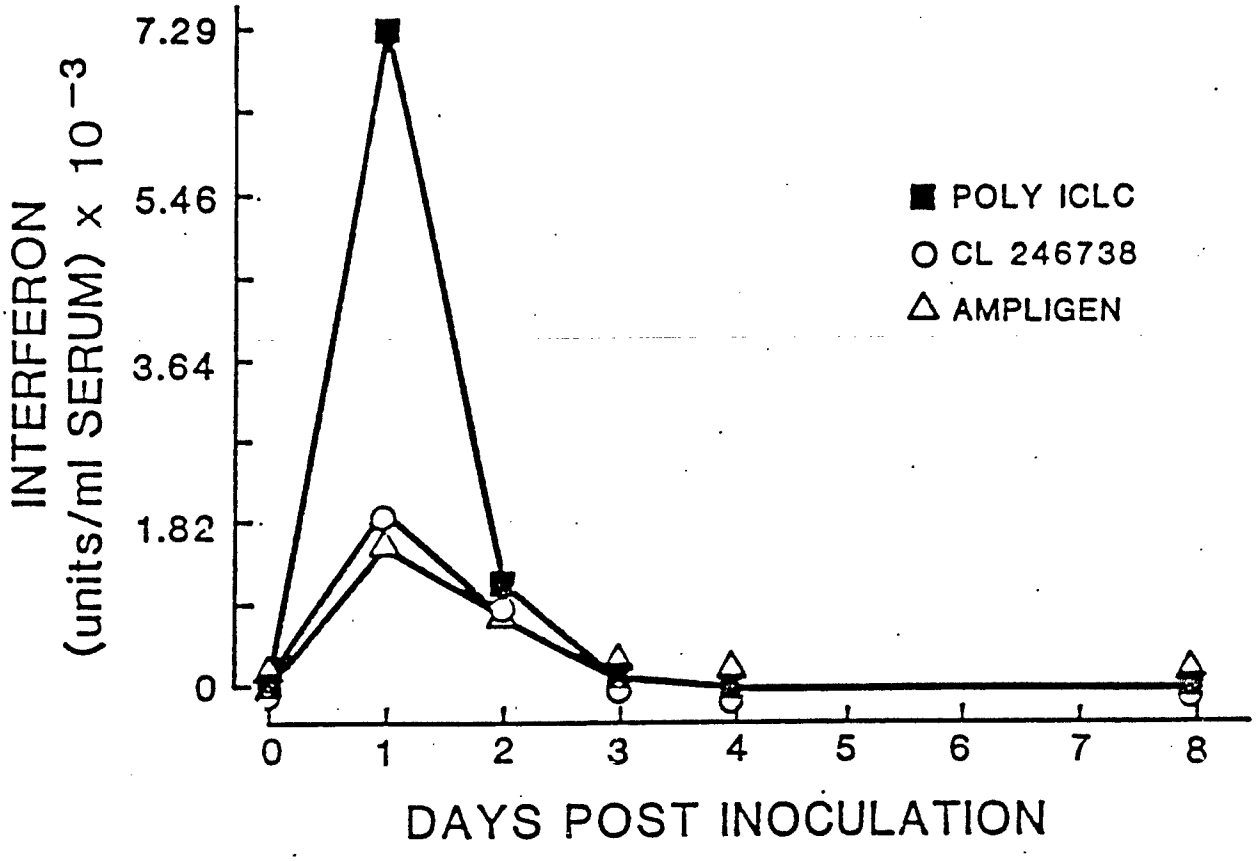

Figure 5. Serum interferon levels at various times after drug treatment. Poly I:C-LC (100 ug) and Ampligen (100 ug) were given iv and CL 246738 (4mg) was given orally. Each point represents the mean of three mice.

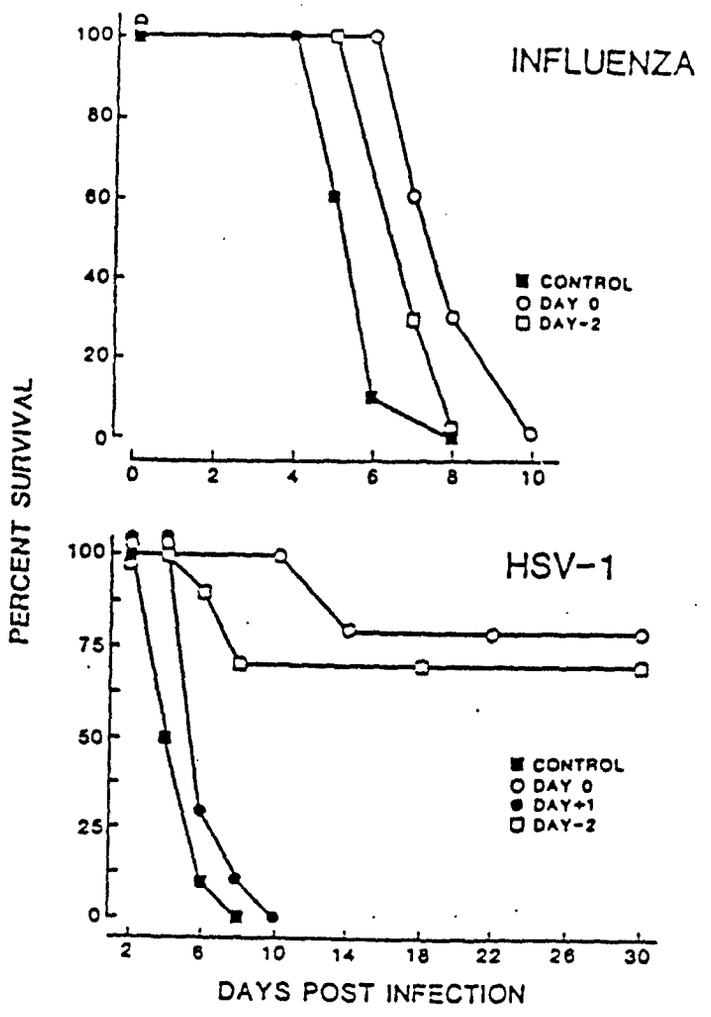

Figure 6. Effect of iv injection of 100 ug Poly I:C-LC on resistance to Influenza or HSV-1 induced pneumonitis. Drug was administered on the days indicated.

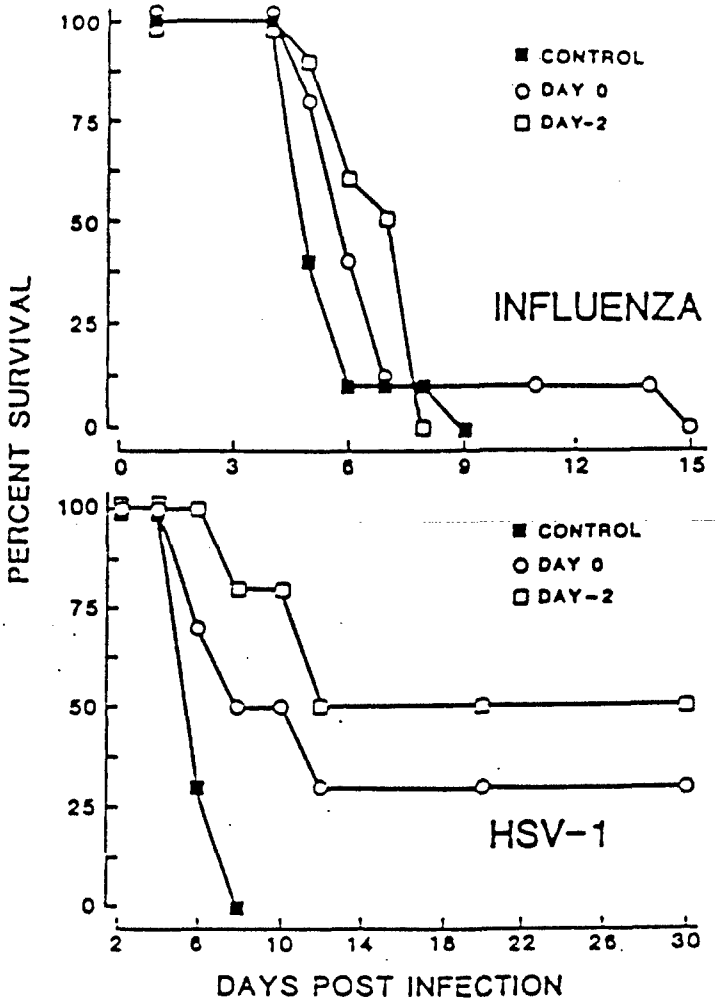

Figure 7. Effect of oral administration of 4mg CL 246738 on resistance to Influenza or HSV-1 induced pneumonitis. Drug was administered on the days indicated.

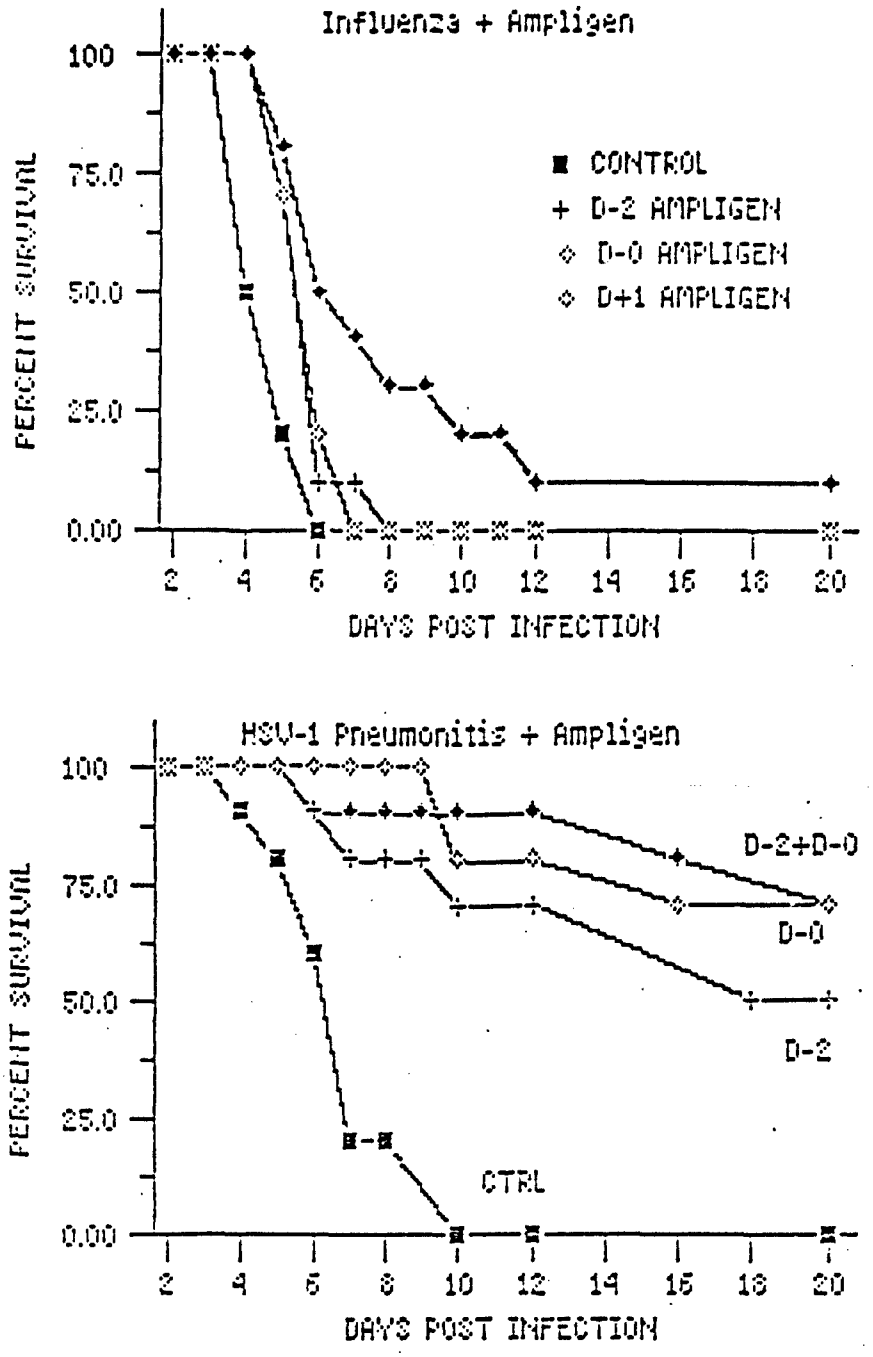

Figure 8. Effect of iv injection of 100 ug Ampligen on resistance to Influenza or HSV-1 induced pneumonitis. Drug was administered on the days indicated.

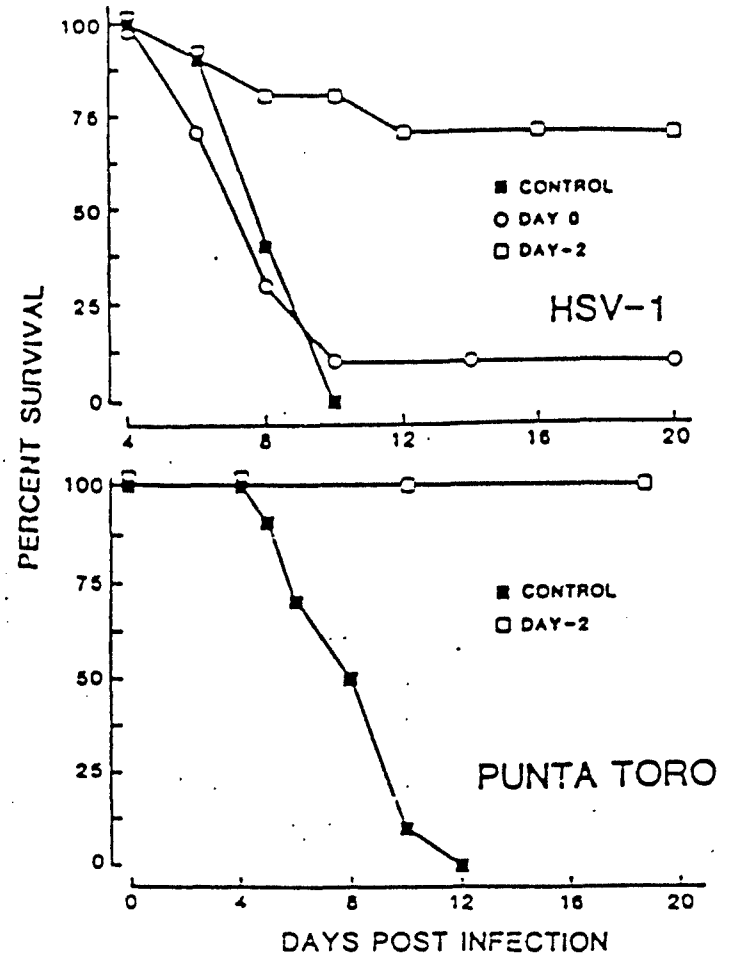

Figure 9. Effect of iv injection of 100 ug Poly I:C-LC on resistance to HSV-1 or Punta Toro induced hepatitis. Drug was administered on the days indicated.

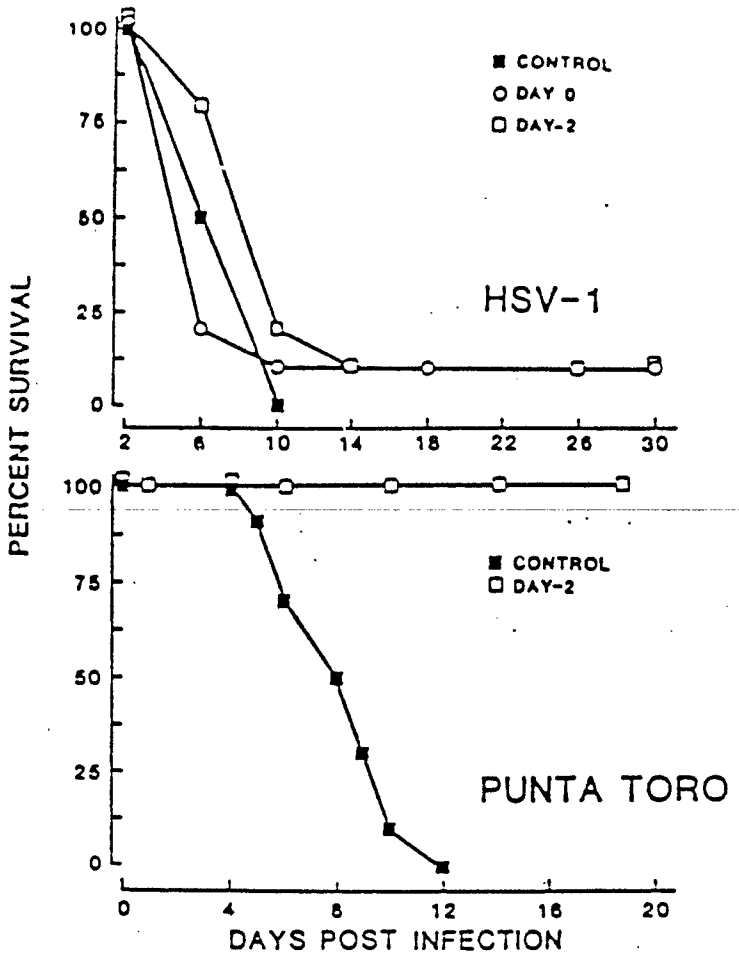

Figure 10. Effect of oral administration of 4mg CL 246738 on resistance to HSV-1 or Punta Toro induced hepatitis. Drug was administered on the days indicated.

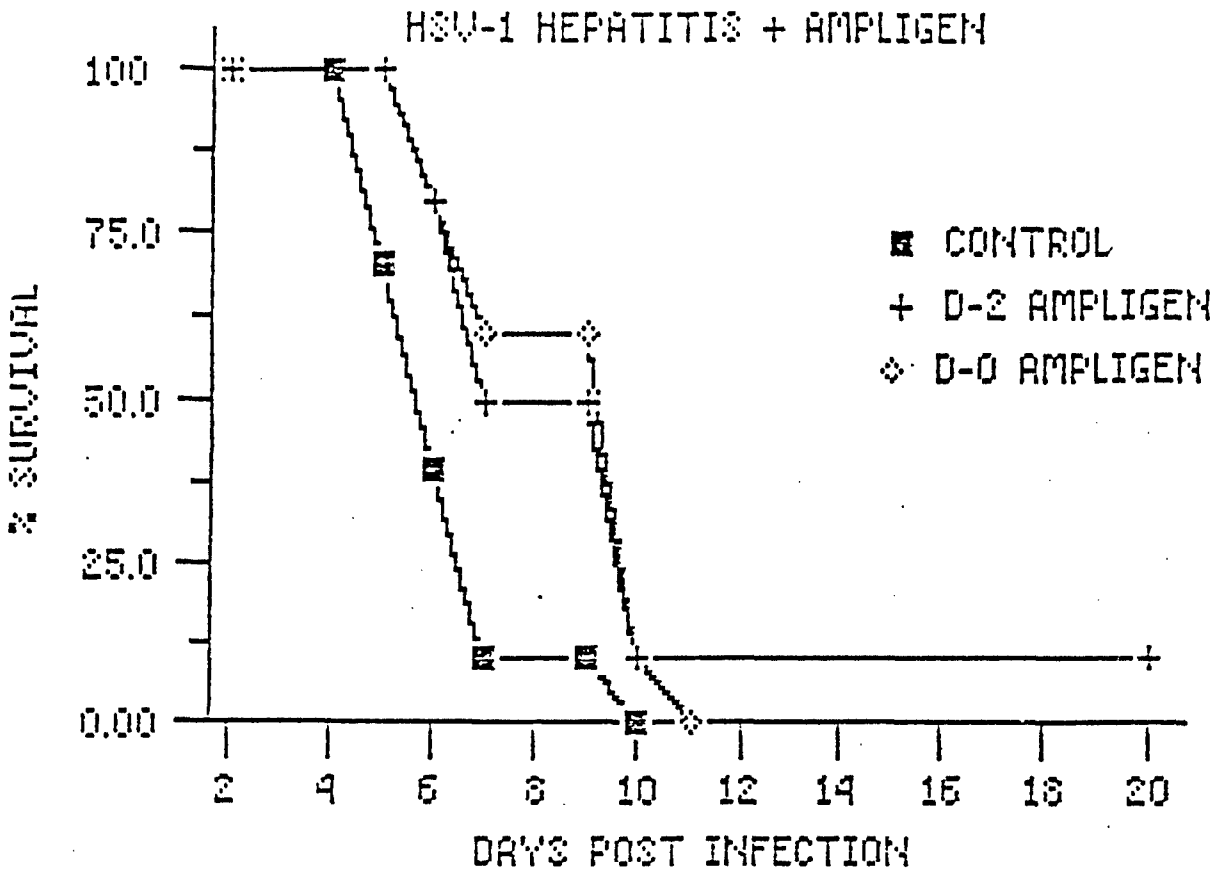

Figure 11. Effect of iv injection of 100 ug Ampligen on resistance to HSV-1 induced hepatitis. Drug was administered on the days indicated.

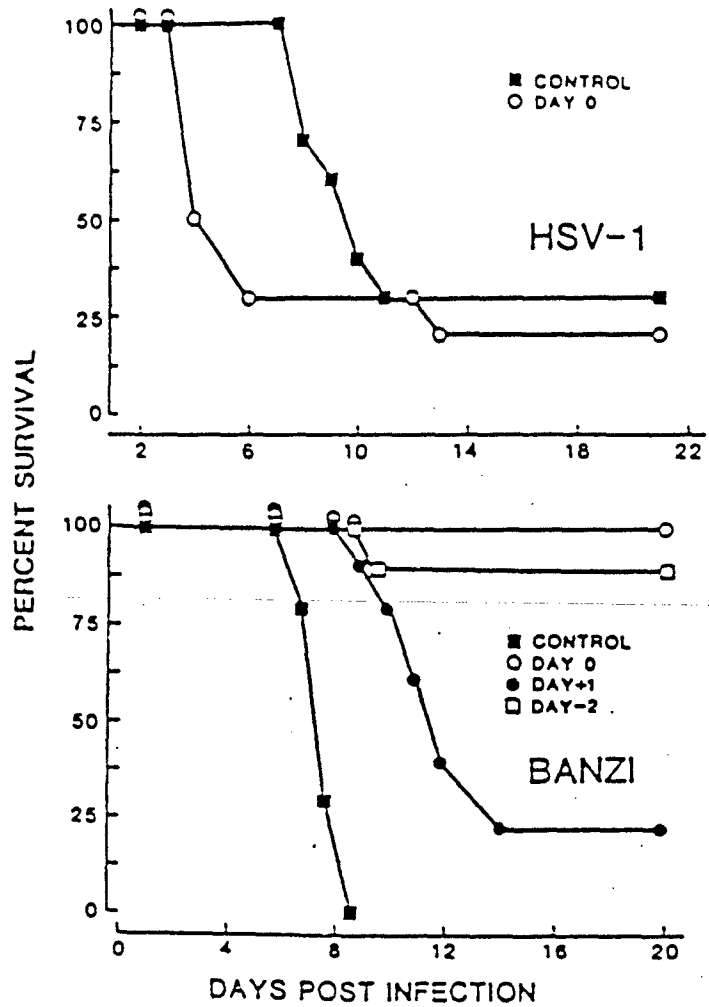

Figure 12. Effect of iv injection of 100 ug Poly I:C-LC on resistance to HSV-1 or Banzi virus induced encephalitis. Drug was administered on the days indicated.

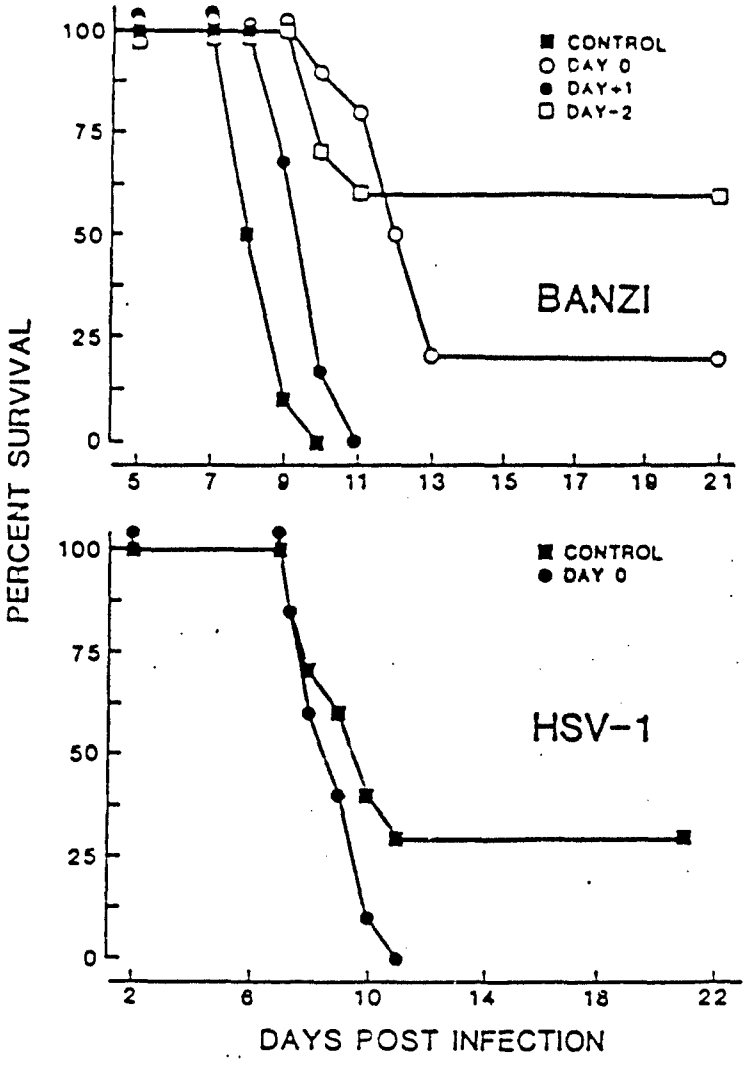

Figure 13. Effect of oral administration of 4mg CL 246738 on resistance to HSV-1 or Banzi virus induced encephalitis. Drug was administered on the days indicated..

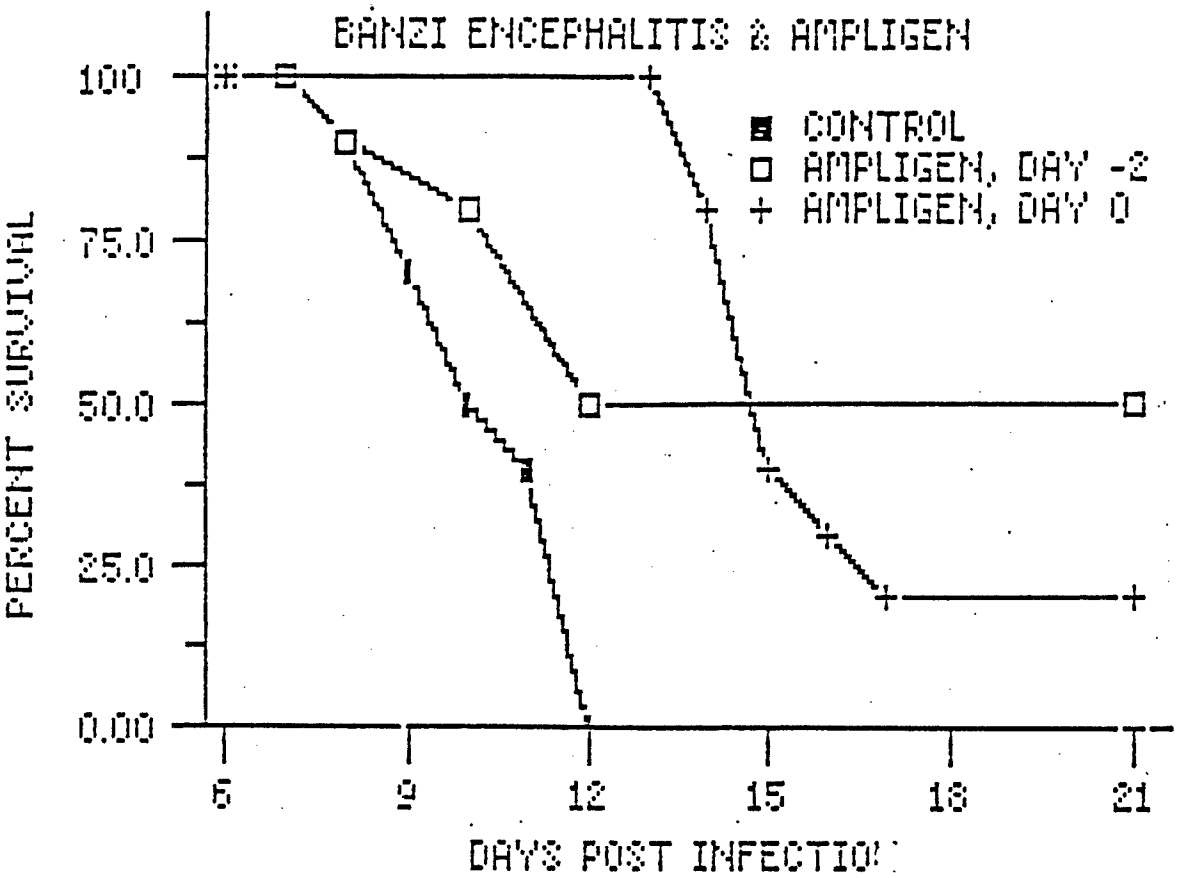

Figure 14. Effect of iv injection of 100 ug Ampligen on resistance to Banzi virus induced encephalitis. Drug was administered on the days indicated.

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